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<b>(21) International Application Number:</b> PCT/US00/10021 <b>(22) International Filing Date:</b> 12 April 2000 (12.04.2000) <b>(30) Priority Data:</b> 60/129,731 16 April 1999 (16.04.1999) US <b>(60) Parent Application or Grant</b> KOSAN BIOSCIENCES, INC. [/]; (). SANTI, Daniel, V. [/]; (). XUE, Qun [/]; (). ASHLEY, Gary [/]; (). MURASHIGE, Kate, H.; ().		<b>Published</b>
<b>(54) Title: A MULTI-PLASMID METHOD FOR PREPARING LARGE LIBRARIES OF POLYKETIDES AND NON-RIBOSOMAL PEPTIDES</b> <b>(54) Titre: METHODE MULTIPLASMIDE DE PREPARATION DE GRANDES BIBLIOTHEQUES DE POLYCETIDES ET DE PEPTIDES NON RIBOSOMIQUES</b>		
<b>(57) Abstract</b> <p>A multiple-plasmid system for heterologous expression of polyketides facilitates combinatorial biosynthesis. The method can be extended to any modular polyketide synthase (PKS) or non-ribosomal peptide synthase (NRPS) and has the potential to produce thousands of novel natural products, including ones derived from further modification of the PKS or NRPS products by tailoring enzymes.</p> <b>(57) Abrégé</b> <p>L'invention concerne un système multiplasmide destiné à l'expression hétérologue des polycétides et qui facilite la biosynthèse combinatoire. Cette méthode peut s'appliquer à n'importe quelle polycétide synthase modulaire (PKS) ou peptide synthase non ribosomique (NRPS), et peut permettre de produire des milliers de nouveaux produits naturels, y compris des produits dérivés des produits PKS ou NRPS modifiés grâce à l'adaptation des enzymes.</p>		

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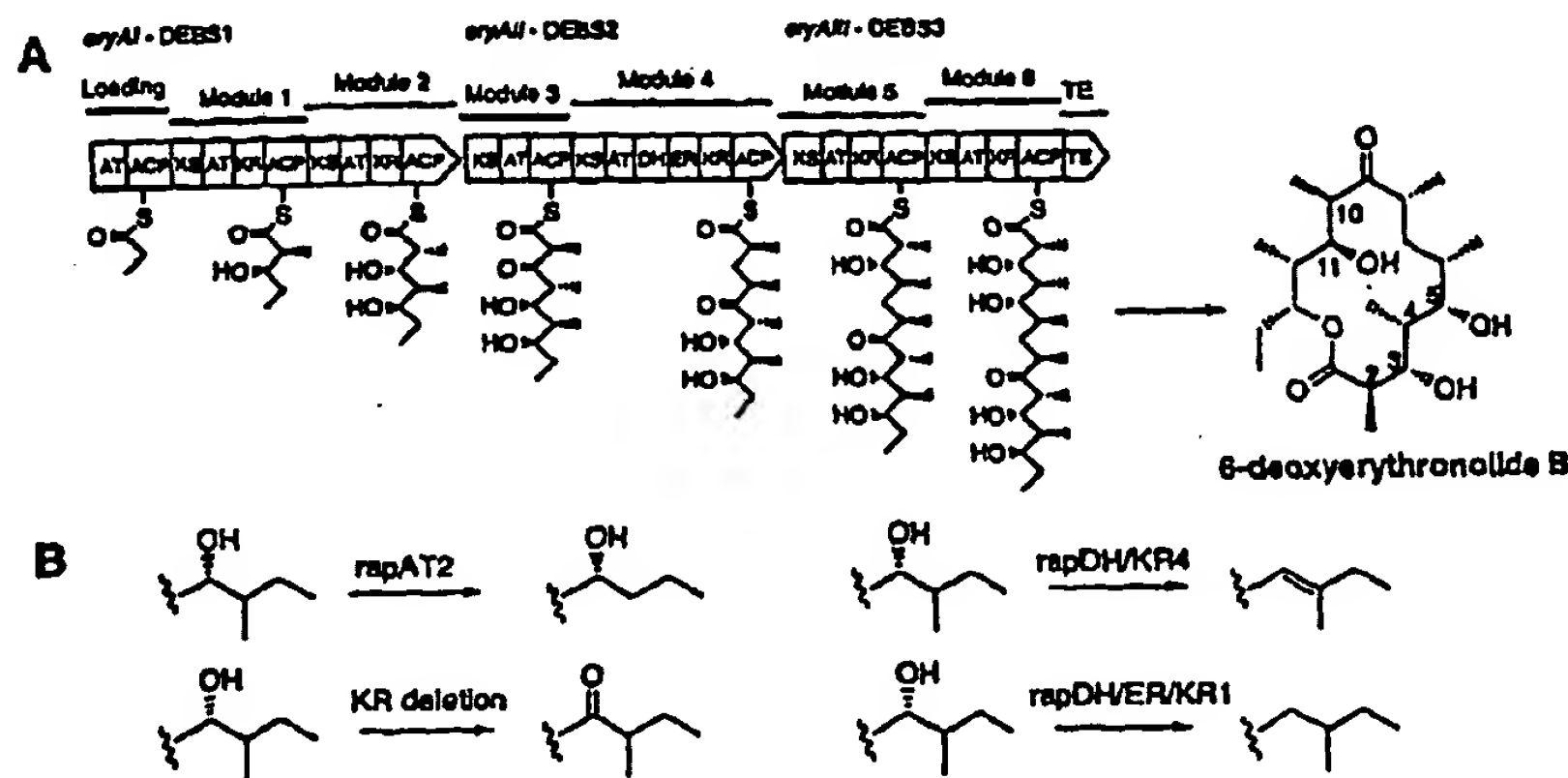
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(21) International Application Number: PCT/US00/10021 (22) International Filing Date: 12 April 2000 (12.04.00)  (30) Priority Data: 60/129,731 16 April 1999 (16.04.99) US  (71) Applicant: KOSAN BIOSCIENCES, INC. [US/US]; 3832 Bay Center Place, Hayward, CA 94545 (US).  (72) Inventors: SANTI, Daniel, V.; 211 Belgrave Avenue, San Francisco, CA 94117 (US). XUE, Qun; 1042 Irving Street, Apt. 4, San Francisco, CA 94112 (US). ASHLEY, Gary; 1102 Verdemar Drive, Alameda, CA 94502 (US).  (74) Agents: MURASHIGE, Kate, H. et al.; Morrison & Foerster LLP, 2000 Pennsylvania Avenue N.W., Washington, DC 20006-1888 (US).		(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  Published <i>Without international search report and to be republished upon receipt of that report.</i>

(54) Title: A MULTI-PLASMID METHOD FOR PREPARING LARGE LIBRARIES OF POLYKETIDES AND NON-RIBOSOMAL PEPTIDES



(57) Abstract

A multiple-plasmid system for heterologous expression of polyketides facilitates combinatorial biosynthesis. The method can be extended to any modular polyketide synthase (PKS) or non-ribosomal peptide synthase (NRPS) and has the potential to produce thousands of novel natural products, including ones derived from further modification of the PKS or NRPS products by tailoring enzymes.

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Title

A Multi-Plasmid Method for Preparing Large Libraries of Polyketides and Non-Ribosomal Peptides

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Reference to Government Funding

This invention was supported in part by SBIR grant 1R43-GM56575-01. The U.S. government has certain rights in this invention.

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Field of the Invention

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The present invention provides recombinant DNA compounds and host cells containing novel polyketide synthase (PKS) genes and novel polyketides. The invention relates to the fields of chemistry, medicinal chemistry, human and veterinary medicine, molecular biology, pharmacology, agriculture, and animal husbandry.

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Background of the Invention

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Polyketides are structurally diverse natural products that include important therapeutic agents used as antibacterials (erythromycin), immunosuppressants (FK506), cholesterol-lowering agents (lovastatin), and others (see Katz *et al.*, 1993, Polyketide synthesis: prospects for hybrid antibiotics, *Annu. Rev. Microbiol.* 47: 875-912, incorporated herein by reference). Currently, there are about 7,000 identified polyketides, but this represents only a small fraction of what nature is capable of producing.

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40

DNA sequencing of genes encoding several of the enzymes that produce type 1 modular polyketide synthases (PKSs) has revealed the remarkably logical organization of these multifunctional enzymes (see Cortes *et al.*, 1990, An unusually large multifunctional polypeptide in the erythromycin-producing polyketide synthase of *Saccharopolyspora erythraea*, *Nature* 348: 176-178; Donadio *et al.*, 1991, Modular organization of genes required for complex polyketide biosynthesis, *Science* 252: 675-679; Schwecke *et al.*, 1995, The biosynthetic gene cluster for the polyketide immunosuppressant rapamycin, *Proc. Natl. Acad. Sci.*

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5 USA 92: 7839-7843; and August *et al.*, 1998, Biosynthesis of the ansamycin  
antibiotic rifamycin: deductions from the molecular analysis of the *rif* biosynthetic  
gene cluster of *Amycolatopsis mediterranei* S699, *Chem Biol* 5: 69-79, each of which is  
10 incorporated herein by reference). The application of innovative combinatorial  
5 techniques to this genetic organization has prompted the generation of novel  
natural products, by adding, deleting, or exchanging domains or entire modules.  
See U.S. Patent Nos. 5,672,491; 5,712,146; 5,830,750; 5,843,718; 5,962,290; and  
15 6,022,731, each of which is incorporated herein by reference. It would be  
advantageous to have a practical combinatorial biosynthesis technology that  
10 could achieve and perhaps exceed the diversity of modular polyketide structures  
thus far revealed in nature.

The known modular PKSs have a linear organization of modules, each of  
which contains the activities needed for one cycle of polyketide chain elongation,  
25 as illustrated for 6-deoxyerythronolide B synthase (DEBS) in Fig. 1A. The minimal  
15 module contains a ketosynthase (KS), an acyltransferase (AT), and an acyl carrier  
protein (ACP) that together catalyze a 2-carbon extension of the chain. The  
specificity of the AT for either malonyl or an alpha-alkyl malonyl CoA determines  
30 which 2-carbon extender is used, and thus the nature of the alkyl substituent at  
the alpha-carbon of the growing polyketide chain. After each 2-carbon unit  
20 condensation, the oxidation state of the beta-carbon is either retained as a ketone,  
35 or modified to a hydroxyl, methenyl, or methylene group by the presence a  
ketoreductase (KR), a KR + a dehydratase (DH), or a KR + DH + an enoyl  
reductase (ER), respectively. In effect, the AT specificity and the composition of  
40 catalytic domains within a module serve as a "code" for the structure of each 2-  
25 carbon unit. The order of the modules in a PKS specifies the sequence of the  
distinct 2-carbon units, and the number of modules determines the size of the  
polyketide chain.

The remarkable structural diversity of polyketides (see O'Hagan, *The*  
*Polyketide Metabolites*; Ellis Horwood, Chichester, 1991, incorporated herein by  
30 reference) is governed by the combinatorial possibilities of arranging modules

5 containing the various catalytic domains, the sequence and number of modules,  
and the post-PKS "tailoring enzymes" that accompany the PKS genes. The direct  
correspondence between the catalytic domains of modules in a PKS and the  
10 structure of the resulting biosynthetic product allows rational modification of  
polyketide structure by genetic engineering.

Over the past several years, examples of modifying each of the elements  
that code for polyketide structure has been accomplished (see Kao *et al.*, 1996,  
15 Evidence for two catalytically independent clusters of active sites in a functional  
modular polyketide synthase, *Biochemistry* 35: 12363-12368; Liu *et al.*, 1997,  
10 Biosynthesis of 2-nor-6-deoxyerythronolide B by rationally designed domain  
substitution, *J. Am. Chem. Soc.* 119: 10553-10554; McDaniel *et al.*, 1997, Gain-of-  
20 function mutagenesis of a modular polyketide synthase, *J. Am. Chem. Soc.* 119:  
4309-4310; Marsden *et al.*, 1998, Engineering broader specificity into an antibiotic-  
25 producing polyketide synthase, *Science* 279: 199-202; and Jacobsen *et al.*, 1997,  
15 Precursor-directed biosynthesis of erythromycin analogs by an engineered  
polyketide synthase, *Science* 277: 367-369, each of which is incorporated herein by  
reference).

Recently, a combinatorial library of over 50 novel polyketides was  
prepared by systematic modification of DEBS, the PKS that produces the  
20 macrolide aglycone precursor of erythromycin (see U.S. patent application Serial  
35 No. 09/429,349, filed 28 Oct. 1999; PCT patent application US99/24483, filed 20  
Oct. 1999; and McDaniel *et al.*, 1999, Multiple genetic modification of the  
erythromycin gene cluster to produce a library of novel "unnatural" natural  
40 products, *Proc. Natl. Acad. Sci. USA* 96: 1846-1851, each of which is incorporated  
25 herein by reference). With a single plasmid containing the *eryAI*, *-AII* and *-AIII*  
genes encoding the three DEBS subunits, ATs and beta-carbon processing  
45 domains were substituted by counterparts from the rapamycin PKS (see Schwecke  
*et al.*, 1995, *supra*) that encode alternative substrate specificities and beta-carbon  
processing activities. The approach used was to develop single "mutations", then  
30 sequentially combine the single mutations to produce multiple changes in the

5 PKS. It was observed that when two or more single PKS mutants were functional,  
there was a high likelihood that combinations would also produce the expected  
polyketide. Although this strategy provided high assurance that the multiple  
10 mutants would be productive, the production of each polyketide required a  
5 separate engineering. Thus, if X mutants of *eryAI*, Y mutants of *eryAII*, and Z  
mutants at *eryAIII* were prepared, X+Y+Z separate experiments were required to  
produce that same number of polyketides. Clearly, the preparation of very large  
15 libraries by this approach is laborious.

Another strategy for preparing large numbers of polyketides is by random  
10 digestion-religation leading to "mutagenesis" of the domains or modules of a  
20 mixture of PKS genes, including the refinements embodied in the DNA shuffling  
method (see Patten *et al.*, 1997, Applications of DNA shuffling to pharmaceuticals  
and vaccines, *Curr. Op. Biotechnol.* 8: 724-733, incorporated herein by reference).  
25 The expected low probability of assembling an active PKS by such an approach,  
15 however, would demand an extraordinary analytical effort (in the absence of a  
biological selection) to detect clones that produced polyketides within the much  
larger number of clones that are non-producers.  
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There remains a need for practical approaches to create large libraries of  
polyketides, non-ribosomal peptides, and mixed polyketides/non-ribosomal  
20 peptides.  
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#### Summary of the Invention

The present invention provides a method for expressing a polyketide or  
40 non-ribosomal peptide in a host cell employing a multiplicity of recombinant  
25 vectors, which may be integrative or freely replicating. Each of the multiplicity of  
vectors encodes a portion of the polyketide synthase or non-ribosomal peptide  
synthase that produces the polyketide or non-ribosomal peptide. In one  
45 embodiment, at least one of the multiplicity of vectors encodes one or more  
proteins that further modify the polyketide or non-ribosomal peptide produced.  
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5 In a preferred embodiment, the vectors replicate in and/or integrate into the chromosome of a *Streptomyces* host cell. Preferred integrating vectors include vectors derived from pSET152 and pSAM2. Preferred replicating vectors include  
10 those containing a replicon derived from SCP2\* or pJV1.

5 In another embodiment, the present invention provides novel polyketides. Such novel polyketides include those shown in Figure 3 as compound nos. 29-43 and 45-59. Other novel polyketides of the invention include the polyketides  
15 obtainable by hydroxylation and/or glycosylation of compounds 29-43 and 45-59. Preferred compounds of the invention include those 14-membered macrolactones  
10 with a C-6 and/or C-12 hydroxyl and/or a C-3 and/or C-5 glycosyl, including but not limited to those with a desosaminyl residue at C-5 and a cladinosyl residue at C-3.  
20

25 These and other embodiments, modes, and aspects of the invention are described in more detail in the following description, the examples, and claims set  
15 forth below.

### 30 Description of the Drawings

Figure 1 shows wild-type and mutant forms of the *eryA* genes and DEBS proteins. Part A depicts the *eryAI-eryAIII* genes and proteins as broad arrows  
20 oriented in the direction of transcription with the domains in modules 1 to 6 of DEBS1-DEBS3 indicated by the symbols defined herein. The first substrate, propionyl-CoA, is attached to the loading domain ACP and (2S)-2-methylmalonyl-CoA to the module 2 ACP. Then, a decarboxylative condensation  
35 between the propionate and methylmalonate takes place followed by reduction of the incipient beta-ketone to form the intermediate shown attached to the ACP of module 2. This intermediate is transferred to the ACP of module 3, and the  
40 sequence of reactions is repeated at each of the other modules with or without ketone reduction, dehydration, or double bond reduction to form the linear 21-carbon polyketide attached to the ACP of module 6. The linear polyketide then is  
45 cyclized and released as 6-deoxyerythronolide B (6dEB). Part B depicts  
30

5 replacement of the one or more of the domains in DEBS1, DEBS2, or DEBS3 with  
one of the three rap (rapamycin) PKS domains or cassettes, or deletion of the KR.  
This results in the corresponding functional group changes shown at one or more  
10 of the positions of 6dEB.

5 Figure 2 shows an illustrative three-plasmid expression system for *eryA*  
genes. In each vector, the *eryA* gene is expressed under the control of the  
upstream *actI* promoter and *actII*-ORF4 gene as previously described (see  
15 Ziermann *et al.*, 2000, A two-vector system for the production of recombinant  
polyketides in *Streptomyces*, *J. Ind. Microbiol. & Biotech.* 24: 46-50; and Kao *et al.*,  
10 1994, Engineered biosynthesis of a complete macrolactone in a heterologous host,  
*Science* 265: 509-512, each of which is incorporated herein by reference). To  
facilitate construction of the various *eryA* mutations, a *SpeI* site (ACTAGT) was  
introduced at nt 10366-10371 of the *eryAI* ORF by making D3455T and A3456S  
25 mutations (see Kao *et al.*, 1995, Manipulation of macrolide ring size by directed  
mutagenesis of a modular polyketide synthase, *J. Am. Chem. Soc.* 117: 9105-9106,  
15 incorporated herein by reference) in pKOS025-179. This change enables insertion  
of the mutated gene segment between the *PacI* site and the *SpeI* site of pKOS025-  
30 179. After the replacement of the 6-kb fragment between the *AscI* sites in *eryAII* (nt  
1213 and nt 7290) with a 6-kb *AscI* fragment containing a specific AT substitution  
20 in an intermediate plasmid, the resulting *PacI*-*XbaI* fragment containing the  
mutant *eryAII* gene was inserted into pKOS025-143. All *eryAIII* mutants were  
35 constructed by replacing the segment in pKOS010-153 between the unique *BglII*  
site at nt 251 and the *EcoRI* site (nt 9290) that overlaps the stop codon.

40 Figure 3 shows structures of macrolactones produced by *Streptomyces*  
25 *lividans* strains containing assorted combinations of three plasmids (Fig. 2 and  
Table 1). The positions in 6dEB (1) that are altered correspond to the genetic  
characteristics of modules 2, 3, 5 and 6 of DEBS, as illustrated in Fig. 1A and Fig. 2,  
45 and listed in Table 1. Structures 1 - 40 and 49 - 56 are 14-membered lactones, and  
structures 44 to 48 are 12-membered lactones. Compounds 49 - 59 with the C13

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propyl group were produced by mutational biosynthesis with the *eryAI* KS1<sup>o</sup> null allele.

#### Detailed Description of the Invention

The present invention provides methods and reagents for using multiple recombinant DNA vectors to produce polyketides in recombinant host cells. In an illustrative embodiment, a three-plasmid system for heterologous expression of 6-deoxyerythronolide B synthase (DEBS) is provided to facilitate combinatorial biosynthesis of polyketides made by type I modular polyketide synthases (PKSs). The *eryA* PKS genes encoding the three DEBS subunits were individually cloned into three compatible *Streptomyces* vectors carrying mutually selectable antibiotic resistance markers. A strain of *Streptomyces lividans* transformed with all three plasmids produced 6-deoxyerythronolide B at a level similar to that of a strain transformed with a single plasmid containing all three genes.

The utility of this system in combinatorial biosynthesis was demonstrated through production of a library of modified polyketide macrolactones, using versions of each plasmid constructed to contain defined mutations. Combinations of these vector sets were introduced into *Streptomyces lividans*, resulting in strains producing a wide range of 6-deoxyerythronolide B analogs. This method can be applied to any modular PKS or non-ribosomal peptide synthase (NRPS) and has the potential to produce thousands of novel natural products, including ones derived from further modification of the PKS or NRPS products by tailoring enzymes.

Thus, in one aspect, the present invention provides a method for using multiple (two, three, four, or more) recombinant DNA vectors, each encoding a portion of a PKS, NRPS, or tailoring enzyme, to produce a polyketide or non-ribosomal peptide or a mixed polyketide/non-ribosomal peptide in a host cell. In one embodiment, the vectors in combination encode a naturally occurring PKS or NRPS and the corresponding natural product is produced. In another embodiment, the vectors in combination encode naturally occurring proteins from two or more different naturally occurring PKS or NRPS. In another embodiment,

5 at least one of the vectors encodes a protein not found in nature, having been altered by recombinant DNA methodology to change its structure and function.

10 In one embodiment, the present invention provides a method for creating a polyketide library that enables the production of large libraries of polyketides while retaining the high probability of obtaining productive clones by combining 5 PKS mutations known to be productive. The principle involves cloning mutants of individual open reading frames (ORFs) of a PKS on separate compatible plasmids, then coexpressing the separate ORFs in a suitable host to produce the PKS. Using 15 this multiple plasmid approach, with X mutants of ORF 1, Y mutants of ORF 2, and Z mutants of ORF 3, for instance, a combinatorial library of  $X \times Y \times Z$  mutants 20 can be achieved expeditiously.

DEBS was chosen to illustrate this multiple plasmid approach. The DEBS 25 PKS consists of three >280 kD protein subunits, each containing two modules, that are assembled into the complete PKS complex (see Donadio *et al.*, 1991, *supra*). 15 With the two possible AT domains and the four possible beta-keto modifications, i.e. eight at each module, therefore  $8 \times 8 = 64$  permutations for the two modules are possible in each DEBS subunit. Constructing the mutations in each DEBS ORF 30 separately would require that 64 manipulations be carried out on each gene, or a total of 192 such manipulations. However, by co-transforming a host strain with 20 three plasmids, each bearing the 64 permutations of a different DEBS subunit, one could generate the mutant PKSs necessary to achieve, in theory, a library of 35 262,144 polyketides ( $64^3$ ), as 6-deoxyerythronolide B (6dEB) analogs (Fig. 1A). In contrast, the same number of mutagenesis experiments performed in a single 40 plasmid system would theoretically yield only 192 polyketides.

25 The successful implementation of this multiple plasmid strategy requires that the DEBS subunits translated from three different mRNAs faithfully interact to give the active PKS. Some indication that this would be the case was provided 45 by *in vitro* experiments that showed that reconstitution of the isolated DEBS1-DEBS2 complex with DEBS3 forms a functional PKS (see Pieper *et al.*, 1995, Cell- 30 free synthesis of polyketides by recombinant erythromycin polyketide synthases,

5 *Nature* 378: 263-266, incorporated herein by reference). Further, it was recently demonstrated that coexpression of the three subunits of DEBS from two plasmids produced active PKS *in vivo* (see Ziermann *et al.*, 2000, *supra*). The present invention can be applied to any PKS or NRPS, including but not limited to the 10 PKS enzymes, including the KS1 null mutation containing versions, that synthesize oleandolide and megalomicin (see U.S. patent application Serial Nos. 60/158,305, filed 8 Oct. 1999 and 09/428,517, filed 28 Oct. 1999, and PCT application No. US99/24478, filed 22 Oct. 1999, each of which is incorporated herein by reference).

10 The method in one embodiment requires at least three vectors that can be separately introduced into a *Streptomyces* or other suitable host strain and concomitantly express functional PKS subunits. Two such vectors are preferred for such purposes: the autonomously replicating SCP2\*-based plasmid pRM1 (see 20 Kao *et al.*, 1994, Engineered biosynthesis of a complete macrolactone in a heterologous host, *Science* 265: 509-512, and U.S. Patent No. 6,022,731, each of which is incorporated herein by reference) and the integrating bacteriophage phiC31-based plasmid pSET152 (see Bierman *et al.*, 1992, Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp., *Gene* 116: 43-49, incorporated herein by reference). Several additional plasmids have 30 been tested as described herein and can be used in accordance with the present invention.

Each of these additional plasmids was tested using constructs that contained identical configurations of an *eryA* gene downstream of the *Streptomyces coelicolor actI* promoter and *actII-ORF4* transcriptional activator as described (see 40 Ziermann *et al.*, 2000, *supra*). Plasmid pB45, a high copy replicating plasmid possessing the pJV1 origin (see Servin-Gonzalez *et al.*, 1995, Sequence and functional analysis of the *Streptomyces phaeochromogenes* plasmid pJV1 reveals a modular organization of *Streptomyces* plasmids that replicate by rolling circle, *Microbiology* 141: 2499-2510, incorporated herein by reference) carrying *eryAII* was 45 introduced into *S. lividans* harboring *eryAI* on pRM1 and *eryAIII* in the pSET152 50

5 integration site; less than 0.1 mg/L of 6dEB was produced in this system compared with 50 mg/L for the single plasmid system (see Kao *et al.*, 1994, *supra*).

10 The multiple vectors used in the present method can include two or more different vectors that share the same origin of replication. For example, two  
5 SCP2\*-type plasmids carrying different antibiotic markers can coexist and express PKS subunits in a *Streptomyces* sp., as has been reported using high-copy number plasmids (see Rajgarhia *et al.*, 1997, Minimal *Streptomyces* sp. strain C5  
15 daunorubicin polyketide biosynthesis genes required for aklanonic acid biosynthesis, *J. Bacteriol.* 179: 2690-2696, incorporated herein by reference). Co-  
10 transformation of *S. lividans* with *eryAIII*/pSET-*apm*, *eryAI*/pRM1-*tsr* and  
20 *eryAII*/pRM1-*hyg* (Fig. 2) yielded a strain that also produced 50 mg/L of 6dEB. Further, after over 24 generations under double antibiotic selection, both  
25 replicating plasmids could be rescued by standard procedures with unchanged restriction maps (see Hopwood *et al.*, 1985, *Genetic Manipulation of Streptomyces. A*  
15 *laboratory manual*. John Innes Foundation, Norwich, incorporated herein by reference).

30 The multiple vectors of the present invention can include two or more different integrating vectors as well. For example, the *eryAII* gene was cloned into a pSAM2 site-specific integrating plasmid (see Smokvina *et al.*, 1990, Construction  
20 of a series of pSAM2-based integrative vectors for use in *Actinomycetes*, *Gene* 94: 53-59, incorporated herein by reference). Sequential transformation of  
35 *Streptomyces lividans* with *eryAIII*/pSET-*apm*, *eryAII*/pSAM-*hyg* and *eryAI*/pRM1-*tsr* provided a strain that produced 40-50 mg/L of 6dEB. A potential advantage of  
40 this system over the two-replicating vector system is that it requires one fewer  
25 antibiotic, and avoids potential problems in maintaining two plasmids containing the same *ori* in a *Streptomyces* host (see Baltz, 1997, *Molecular genetic approaches to yield improvement in Actinomycetes, Biotechnology of Antibiotics*, 2nd. Edition: pp. 49-62, incorporated herein by reference). However, as shown herein, the two  
45 SCP2\*-based plasmids and the pSET-derived vector can also be used.

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5                   Thus, the invention can be practiced with a wide variety of expression  
vectors for use in *Streptomyces*. The replicating expression vectors of the present  
invention include, for example and without limitation, those that comprise an  
10                   origin of replication from a low copy number vector, such as SCP2\* (see  
5                   Hopwood *et al.*, *Genetic Manipulation of Streptomyces: A Laboratory manual* (The  
John Innes Foundation, Norwich, U.K., 1985); Lydiate *et al.*, 1985, *Gene* 35: 223-235;  
and Kieser and Melton, 1988, *Gene* 65: 83-91, each of which is incorporated herein  
15                   by reference), SLP1.2 (Thompson *et al.*, 1982, *Gene* 20: 51-62, incorporated herein  
by reference), and pSG5(ts) (Muth *et al.*, 1989, *Mol. Gen. Genet.* 219: 341-348, and  
10                   Bierman *et al.*, 1992, *Gene* 116: 43-49, each of which is incorporated herein by  
reference), or a high copy number vector, such as pIJ101 and pJV1 (see Katz *et al.*,  
20                   1983, *J. Gen. Microbiol.* 129: 2703-2714; Vara *et al.*, 1989, *J. Bacteriol.* 171: 5782-5781;  
and Servin-Gonzalez, 1993, *Plasmid* 30: 131-140, each of which is incorporated  
25                   herein by reference). High copy number vectors are, however, generally not  
15                   preferred for expression of large genes or multiple genes. For non-replicating and  
integrating vectors and generally for any vector, it is useful to include at least an  
30                   *E. coli* origin of replication, such as from pUC, p1P, p1I, and pBR. For phage based  
vectors, the phage phiC31 and its derivative KC515 can be employed (see  
Hopwood *et al.*, *supra*). Also, plasmid pSET152, plasmid pSAM, plasmids pSE101  
20                   and pSE211, all of which integrate site-specifically in the chromosomal DNA of *S.*  
35                   *lividans*, can be employed for purposes of the present invention.

                  Moreover, a wide variety of selectable markers can be employed in the  
*Streptomyces* recombinant expression vectors of the invention. These include  
40                   antibiotic resistance conferring genes selected from the group consisting of the  
25                   *ermE* (confers resistance to erythromycin and lincomycin), *tsr* (confers resistance  
to thiostrepton), *aadA* (confers resistance to spectinomycin and streptomycin),  
45                   *aacC4* (confers resistance to apramycin, kanamycin, gentamicin, geneticin (G418),  
and neomycin), *hyg* (confers resistance to hygromycin), and *vph* (confers resistance  
to viomycin) resistance conferring genes. Alternatively, several polyketides are



naturally colored, and this characteristic can provide a built-in marker for identifying cells.

An illustrative library of the present invention was constructed. The library was composed of vectors encoding three single mutations in *eryAI* (module 2), one in *eryAII* (module 3), and seven in *eryAIII* (modules 5 or 6) as well as wild-type ORFs, a KS1 null mutant, and a module 6 deletion, as shown in Table 1, below. To facilitate cloning, vectors were prepared that contained restriction sites that allowed transfer of DNA cassettes from previously prepared mutant *eryA* genes (see McDaniel *et al.*, 1999, *supra*, and Fig. 2). Fourteen of these expression vectors, comprising the three wild-type and eleven mutant ORFs were constructed by cassette transfers from plasmids previously prepared in the single plasmid system.

Table 1

Genotype of the plasmids containing DEBS genes<sup>a</sup>

Vector:	pKOS021	pKOS025	pKOS010
Gene:	<i>eryAI</i> (DEBS1)	<i>eryAII</i> (DEBS2)	<i>eryAIII</i> (DEBS3)
Module:	1	3	5 6
1. wild-type			
2. AT-> <sup>b</sup> rapAT2			
3. KR->rapDH/KR4			
4. KR->rapDH/ER/KR1			
5. KS1 <sup>c</sup>			
6. wild-type			
7. AT3->rapAT2			
8. wild-type			
9. AT->rapAT2			
10. KR->AT/ACP linker			
11. KR->rapDH/KR4			
12. KR->rapDH/ER/KR1			
13. module 5 + TE <sup>d</sup>			
14. AT->rapAT2			
15. KR->AT/ACP linker			
16. KR->rapDH/KR4			

<sup>a</sup> The plasmids with mutant DEBS genes were constructed by cloning the specified DNA segments into one of the three vectors shown in Fig. 2.

<sup>b</sup> The arrow signifies that the wild-type domain was replaced with the one indicated.

<sup>c</sup> The Cys729Ala null allele was created by site-specific mutagenesis.

<sup>d</sup> Module 6 was deleted to fuse its TE to the ACP of module 5.



5 In *eryAI*, module 2 was modified by replacing the AT by rapAT2 (the AT domain of module 2 of the rapamycin PKS), and the KR by rapDH/KR4 (the DH and KR domains of module 4 of the rapamycin PKS) or rapDH/ER/KR1 (the DH, 10 KR, and ER domains of module 1 of the rapamycin PKS). In *eryAII*, the AT of module 3 was replaced by rapAT2; in *eryAIII*, module 5 was modified by replacing the AT by rapAT2, and the KR by rapDH/KR4 or rapDH/ER/KR1 or the AT/ ACP linker that eliminates the *ery* module 5 KR activity. Also, in module 15 6 the AT was replaced with rapAT2, and the KR by rapDH/KR4 or the AT/ ACP linker. The consequence of introducing each of these rapamycin PKS gene cassettes into DEBS is shown in Fig. 1B; at a given alpha-position in the growing 20 polyketide carbon chain, a methyl group can be removed, or a beta-hydroxyl can be changed to a ketone or removed by dehydration to produce a double bond, or the double bond resulting from dehydration can then be reduced to a methylene group.

25 The approach employed was to first introduce the eight *eryAIII* variants individually into the pSET integration site of *S. lividans*, then to cotransform the resulting strains individually with each of four *eryAI* variants on SCP2<sup>+</sup>-*tsr* vectors 30 and two *eryAII* variants on SCP2<sup>+</sup>-*hyg* vectors. Thus, from the 14 vectors prepared, 64 triple transformants were obtained. Recombinant cells were grown under appropriate antibiotic selection, and extracts analyzed for polyketide production 35 by LC/MS. Of the 64 triple transformants, 46 (72%) produced detectable levels of one or more polyketides under the test conditions employed (Fig. 3), and 43 different polyketides were produced. These included 6dEB (1), and products 40 arising from 11 single (2-12), 26 double (13-38) and five (39-43) triple mutants of DEBS. Twenty-eight (1-28) of the 43 polyketides produced have been previously 25 prepared using the single-plasmid system. Fifteen (29-43) were novel polyketides readily identifiable by mass spectra and correspondence to the products expected 45 based on the cassettes used in the mutagenesis.

In one aspect, the present invention provides these novel polyketides. 50 These novel polyketides can be further modified by tailoring enzymes, including

5 the tailoring enzymes of *Saccharopolyspora erythraea*. There are a wide variety of  
diverse organisms that can modify macrolide aglycones to provide compounds  
with, or that can be readily modified to have, useful activities. For example,  
10 *Saccharopolyspora erythraea* can convert 6-dEB or derivatives thereof to a variety of  
5 useful compounds. The erythronolide 6-dEB is converted by the *eryF* gene  
product to erythronolide B, which is, in turn, glycosylated by the *eryB* gene  
product to obtain 3-O-mycarosylerythronolide B, which contains L-mycarose at C-  
15 3. The enzyme *eryC* gene product then converts this compound to erythromycin D  
by glycosylation with D-desosamine at C-5. Erythromycin D, therefore, differs  
10 from 6-dEB through glycosylation and by the addition of a hydroxyl group at C-6.  
20 Erythromycin D can be converted to erythromycin B in a reaction catalyzed by the  
*eryG* gene product by methylating the L-mycarose residue at C-3. Erythromycin D  
is converted to erythromycin C by the addition of a hydroxyl group at C-12 in a  
25 reaction catalyzed by the *eryK* gene product. Erythromycin A is obtained from  
15 erythromycin C by methylation of the mycarose residue in a reaction catalyzed by  
the *eryG* gene product. The aglycone compounds provided by the present  
invention, such as, for example, the compounds produced in *Streptomyces lividans*,  
30 can be provided to cultures of *S. erythraea* and converted to the corresponding  
derivatives of erythromycins A, B, C, and D. To ensure that only the desired  
20 compound is produced, one can use an *S. erythraea eryA* mutant that is unable to  
35 produce 6-dEB but can still carry out the desired conversions (Weber *et al.*, 1985, *J.*  
*Bacteriol.* 164(1): 425-433). Also, one can employ other mutant strains, such as *eryB*,  
*eryC*, *eryG*, and/or *eryK* mutants, or mutant strains having mutations in multiple  
40 genes, to accumulate a preferred compound. The conversion can also be carried  
25 out in large fermentors for commercial production.

Moreover, there are other useful organisms that can be employed to  
45 hydroxylate and/or glycosylate the compounds of the invention. As described  
above, the organisms can be mutants unable to produce the polyketide normally  
produced in that organism, the fermentation can be carried out on plates or in  
30 large fermentors, and the compounds produced can be chemically altered after

5 fermentation. Thus, *Streptomyces venezuelae*, which produces picromycin, contains  
enzymes that can transfer a desosaminyl group to the C-5 hydroxyl and a  
hydroxyl group to the C-12 position. In addition, *S. venezuelae* contains a  
10 glucosylation activity that glucosylates the 2'-hydroxyl group of the desosamine  
5 sugar. This latter modification reduces antibiotic activity, but the glucosyl residue  
is removed by enzymatic action prior to release of the polyketide from the cell.  
15 Another organism, *S. narbonensis*, contains the same modification enzymes as *S.*  
*venezuelae*, except the C-12 hydroxylase. Thus, the present invention provides the  
compounds produced by hydroxylation and glycosylation of the macrolide  
10 aglycones of the invention by action of the enzymes endogenous to *S. narbonensis*  
20 and *S. venezuelae*.

Other organisms suitable for making compounds of the invention include  
*Micromonospora megalomicea*, which produces megalomicin A. Megalomicin A  
25 contains the complete erythromycin C structure, and its biosynthesis also involves  
15 the additional formation of megosamine (L-rhodosamine) and its attachment to  
the C-6 hydroxyl, followed by acylation of the C-3''' and(or) C-4''' hydroxyls as  
the terminal steps. Other organisms useful in converting the aglycones of the  
30 present invention to modified compounds include *Streptomyces antibioticus*, *S.*  
*fradiae*, and *S. thermotolerans*. *S. antibioticus* produces oleandomycin and contains  
20 enzymes that hydroxylate the C-6 and C-12 positions, glycosylate the C-3  
35 hydroxyl with oleandrose and the C-5 hydroxyl with desosamine, and form an  
epoxide at C-8-C-8a. *S. fradiae* contains enzymes that glycosylate the C-5 hydroxyl  
with mycaminose and then the 4'-hydroxyl of mycaminose with mycarose,  
40 forming a disaccharide. *S. thermotolerans* contains the same activities as *S. fradiae*,  
25 as well as acylation activities. Thus, the present invention provides the  
compounds produced by hydroxylation and glycosylation of the macrolide  
aglycones of the invention by action of the enzymes endogenous to *S. antibioticus*,  
45 *S. fradiae*, and *S. thermotolerans*. Moreover, these and other tailoring enzymes can  
be cloned and incorporated into one or more of the multiple vectors used in  
50

5 accordance with the methods of the present invention to create libraries of modified polyketide compounds.

10 The resulting compounds can be further modified by synthetic chemistry, i.e., to yield the corresponding ketolides, useful as antibiotics (see, e.g., U.S. patent application Serial Nos. 60/172,159, filed 17 Dec. 1999; 60/140,175, filed 18 June 1999; 60/172,154, filed 17 Dec. 1999; and 60/129,729, filed 16 Apr. 1999, each of which is incorporated herein by reference), or to yield the corresponding motilides (see U.S. provisional patent application Serial No. 60/183,338, filed 18 Feb. 2000, attorney docket no. 30062-30053.00, inventors G. Ashley *et al.*, 15 incorporated herein by reference).

20 In 43 of the 46 transformants that produced polyketides in the illustrative library described herein, the isolated polyketides had structures expected of the mutation(s). However, as observed in corresponding single mutations of the *eryA* gene in the single-plasmid system, additional products were observed with 25 certain mutations. In most cases, the rapAT2 domain in module 3 recognized and processed both malonyl- and methylmalonyl-CoA and gave the expected 8-nor analogs plus lesser amounts of the 8-methyl analogs. In a few cases, only the 8-methyl analogs were formed. The relaxed-specificity of rapAT2 appears to be module-dependent, because only the expected products were observed with the 30 same rapAT2 sequence at modules 2 or 5 (see Ruan *et al.*, 1997, Acyltransferase domain substitutions in erythromycin polyketide synthase yield novel erythromycin derivatives, *J. Bacteriol.* 179: 6416-25, incorporated herein by reference).

40 Likewise, when the KR of module 5 was replaced by either the rapDH/KR4 or rapDH/ER/KR1 domains to give the expected 4,5-anhydro and 5-deoxy analogs, respectively, 5-keto analogs were formed in addition to the 45 expected products. This possibly results from transfer of the beta-ketothioester intermediate from KR5 to ACP5 at a rate competitive with its reduction by the heterologous rapKR4 domain. Because aberrant products were not observed with 50

5 rapDH/KR4 at modules 2 or 6, the non-specificity appears to be module-  
dependent.

10 Finally, when the KR of module 2 was replaced with the rapDH/ER/KR1  
domain to provide the 11-deoxy analogs, the 10,11-dehydro analogs were often  
5 observed as minor but significant products. Here, the intermediate is processed by  
the heterologous rapKR and DH domains in module 2, but transfer of the 10,11-  
15 dehydro intermediate to KS3 must be competitive with the ER-catalyzed  
reduction. Interestingly, with rapDH/ER/KR1 in module 2 and rapAT2 in  
module 3, the 10,11-dehydro by-products were not detected. Either the levels  
10 produced were too low for detection using the methods employed, or the aberrant  
20 dehydro intermediate of module 2 was not processed by module 3 containing the  
rapAT2 substitution.

25 Of the 18 transformants that did not produce polyketides at levels  
detectable in these experiments, two were double mutants and 16 were triple  
15 mutants; only one of these mutants was previously prepared in the single-plasmid  
system where it also failed to produce a detectable polyketide. As previously  
30 reported with the single-plasmid system, an increased number of mutations  
resulted in a decrease in yield to a level undetectable by the analytical method  
used.

35 A major advantage of the multiple plasmid system is that once multiple  
plasmids encoding functional mutants of PKS subunits are available, they can be  
rapidly combined with one or more additional mutants to expand the library of  
polyketides. In one example, a single Cys729Ala mutation at the KS1 domain of  
40 DEBS1 module 1 (see Jacobsen *et al.*, 1997, *supra*) was prepared, yielding the KS1  
25 null mutation. The inactive KS1 prevents propagation of the starter unit and  
permits introduction of exogenous synthetic diketide thiol esters into positions 12  
and 13 of the 14-membered macrolide product. This system was first developed  
45 using a single vector, plasmid pJRJ2. Plasmid pJRJ2 encodes the *eryAI*, *eryAII*, and  
*eryAIII* genes; the *eryAI* gene contained in the plasmid contains the KS1 null  
30 mutation. The KS1 null mutation prevents formation of the 6-deoxyerythronolide

5 B produced by the wild-type gene unless exogenous substrate is provided.  
Plasmid pJRJ2 and a process for using the plasmid to prepare novel 13-substituted  
erythromycins are described in PCT publication Nos. 99/03986 and 97/02358 and  
10 in U.S. patent application Serial Nos. 08/675,817, filed 5 July 1996; 08/896,323,  
5 filed 17 July 1997; and 09/311,756, filed 14 May 1999, each of which is  
incorporated herein by reference. The exogenous substrates provided can be  
15 prepared by the methods and include the compounds described in PCT patent  
application No. PCT/US00/02397 (Attorney Docket No. 30062-20032.40) and U.S.  
patent application Serial No. 09/492,733 (Attorney Docket No. 30062-20032.00),  
10 both filed 27 Jan. 2000, by inventors G. Ashley et al., and both of which claim  
20 priority to U.S. patent application Serial No. 60/117,384, filed 27 Jan. 1999, each of  
which is incorporated herein by reference.

The plasmid encoding the KS1 null allele of *eryAI* (Table 1) was introduced  
25 by co-transformation into *Streptomyces lividans* with the one *eryAII* mutant and  
15 seven *eryAIII* mutants (Table 1) to provide 16 transformants. Treatment of each of  
these with a propyl-diketide *N*-acetylcysteine thioester, following the work of  
30 Jacobsen *et al.*, 1997, and the methodology of the patent applications, *supra*,  
provided eleven novel 13-propyl polyketide analogs (49-59, Fig. 3). Thus, the  
present invention provides these novel polyketides as well as the compounds  
20 resulting from their modification by post-PKS tailoring enzymes (oxidases and  
35 glycosylases) or by synthetic chemical methods. To prepare these same PKSs by  
the single-plasmid system would have required preparation of 16 individual  
mutants rather than the single KS1 null mutant used in the present method.

40 In another example, the variants of *eryAI* and *eryAII* were used to prepare a  
25 small library of 12-membered macrolactones. It was previously shown in the *eryA*  
single-plasmid system that omission of module 6, along with fusion of module 5  
45 to the thioesterase domain of DEBS3 (Fig. 1A) results in formation of a 12-  
membered macrolactone. A truncated *eryAIII* gene containing module 5-TE (see  
Kao *et al.*, 1995, Manipulation of macrolide ring size by directed mutagenesis of a  
50 modular polyketide synthase, *J. Am. Chem. Soc.* 117: 9105-9106, incorporated

5 herein by reference, and Table 1) was introduced into *Streptomyces lividans*, and  
the strain transformed with permutations of the four *eryAI* and two *eryAII*  
variants described above. Of the eight 12-membered lactones that could have been  
10 produced, five were observed (44-48). Compound 44 results from combining the  
wild-type *eryAI* and *eryAII* genes with the module 5-TE construct, and has  
5 previously been prepared in the single-plasmid system; compounds 45-48 are  
novel products that more than double the number of known 12-membered  
15 macrolides. These novel macrolactones constitute an important aspect of the  
present invention.

10 Other uses of this multiple plasmid system are as follows. The expression  
and genetic engineering of very large PKS genes, such as those involved in the  
biosynthesis of rapamycin (14 modules) or rifamycin (10 modules), is readily  
20 achievable by this method. The ORFs for each of the subunits of these and other  
PKSs, such as the mixed NRPS-PKS for epothilone (see U.S. patent application  
25 Serial No. 09/443,501, filed 19 Nov. 1999, and PCT patent application  
US99/27438, filed 19 Nov. 1999, each of which is incorporated herein by  
reference) could be cloned and expressed in the manner used here to greatly  
30 simplify genetic manipulations and the structure/function analysis. Thus, in this  
application of the present method, a naturally occurring polyketide can be  
20 expressed in a recombinant host cell.

35 Similarly, it would be desirable to mix PKS genes from entirely different  
pathways to facilitate production of heterologous and hybrid PKSs, as  
precedented by the work of Li *et al.* involving hybrid erythromycin/picromycin  
40 and oleandomycin/picromycinPKS genes (see U.S. patent application Serial Nos.  
25 09/320,878, filed 27 May 1999, and 09/428,517, filed 28 Oct. 1999; PCT patent  
publication No. 99/61599; and PCT patent application No. US99/24478, each of  
45 which is incorporated herein by reference). Such work could include extending  
the carbon chain by the addition of modules.

The PKS libraries generated could be leveraged and expanded by  
50 30 introducing genes for tailoring enzymes that oxidize, hydroxylate, methylate,



5 acylate, glycosylate, or otherwise modify the product of the PKS or a modified  
polyketide, and genes encoding such enzymes could be employed using an  
additional vector in the multiple vector system. Various tailoring enzymes from  
10 different host organisms could be employed in the same system. Genes for such  
5 tailoring enzymes can be obtained as described in the patent applications and  
publications referenced in the preceding paragraph and elsewhere herein. See  
also, U.S. Patent No. 5,998,194, incorporated herein by reference.

15 Finally, the method should be useful with any system consisting of  
multimodular proteins, such as the large family of non-ribosomal peptide  
10 synthases, which produce many pharmaceutically important compounds such as  
20 anti-fungal compounds, anti-cancer compounds, and antibiotics. These important  
compounds are made by multifunctional synthetases, consisting of complexes of  
proteins containing between one and eleven modules, comparable to the modular  
25 PKSs (see Konz *et al.*, 1999, How do peptide synthase generate structural  
15 diversity? *Chem. & Biol.* 6: 39-48, incorporated herein by reference).

The multi-plasmid technology enables the realization of the full potential of  
30 modular PKSs and NRPSs, and thus libraries containing a complete repertoire of  
polyketides and non-ribosomal peptides. The achievement of this objective  
requires only the construction of a limited number of highly expressing,  
20 productive single mutants that will assure adequate polyketide production when  
35 the mutations are combined. Because all the elements for producing an  
extraordinarily large polyketide library are contained within this facile system,  
there is neither need nor benefit to embark on developing more complicated, less  
40 reliable systems to reach the same objective.

25 The following examples are given for the purpose of illustrating the  
present invention and shall not be construed as being a limitation on the scope of  
45 the invention or claims.



Example 1Construction of Expression Plasmids

In each vector, the *eryA* gene is expressed under the control of the upstream *actI* promoter and *actII*-ORF4 gene as previously described (see U.S. Patent No. 5,672,491 and Ziermann *et al.*, 2000, *supra*, both of which are incorporated herein by reference). For testing the high copy relicating plasmid possessing the pJV1 origin, the pB45 based *E. coli* shuttle vector pKOS025-32 was constructed by fusing pB45 with litmus 28 (New England Biolabs) at *Bgl*III and *Pst*I sites. Into pKOS025-32 the ca. 14-kb *Hind*III-*Xba*I fragment containing the *actI* promoter and *actII*-ORF4 gene was inserted followed by the *eryAII* gene to give pKOS025-35. The configuration of the components in the *Hind*III-*Xba*I fragment is shown in Fig. 2. The same *Hind*III-*Xba*I fragment was also cloned into shuttle vector pOSint1/Hygro (see Raynal *et al.*, 1998, Structure of the chromosomal insertion site for pSAM2: functional analysis in *Escherichia coli*, *Molecular Microbiology* 28: 333-342, incorporated herein by reference) to yield the *eryAII*/pSAM2-hyg plasmid, pKOS038-67.

Example 2Transfer of Mutation Cassettes into the Three-plasmid System

The plasmids containing *eryAI* mutations in module 2 were pKOS025-179 (AT2→rapAT2), pKOS038-1 (KR→rapDH/KR4) and pKOS038-3 (KR→rapDH/ER/KR1) and were made as follows. The *Pac*I-*Spe*I fragments containing the corresponding mutations were transferred into the plasmids described in Fig. 2 from plasmids pKOS008-41, pKOS015-56 and pKOS015-57, respectively (see McDaniel *et al.*, 1999, *supra*, and U.S. patent application Serial No. 09/429,349, filed 28 Oct. 1999, and PCT patent application US99/24483, filed 20 Oct. 1999, each of which are incorporated herein by reference). The plasmids containing the *eryAIII* mutations in module 5 were pKOS025-1831 (AT→rapAT2), pKOS025-1832 (KR→AT/ACP linker), pKOS025-1833 (KR→rapDH/KR4) and pKOS025-1834 (KR→rapDH/ER/KR1); and in module 6 were pKOS025-

5 1841(KR→ rapDH/KR4), pKOS021-106 (KR→AT/ ACP linker) and pKOS025-1842  
(AT→rapAT2). These were constructed as follows. The BglIII-EcoRI fragments  
containing the corresponding mutations were transferred into the plasmids  
10 described in Fig. 2 from plasmids pKOS006-188, pKOS016-12, pKOS006-178,  
5 pKOS026-11b, pKOS011-25, pKOS011-13 and pKOS015-53, respectively (see  
McDaniel et al., 1999, *supra*, and U.S. patent application Serial No. 09/429,349,  
filed 28 Oct. 1999, and PCT patent application US99/24483, filed 20 Oct. 1999,  
15 each of which are incorporated herein by reference). Plasmid pKOS038-20  
contains *eryAII* with the AT3→ rapAT2 replacement in module 3 and was made  
10 by transferring the mutation from a previously prepared sub-clone pKOS015-28  
20 (see McDaniel et al., 1999, *supra*, and U.S. patent application Serial No. 09/429,349,  
filed 28 Oct. 1999, and PCT patent application US99/24483, filed 20 Oct. 1999,  
each of which are incorporated herein by reference).

15

### Example 3

#### Streptomyces Transformation

30 *Streptomyces lividans* K4-114 and K4-155 (see U.S. patent application Serial  
No. 09/181,833, filed 28 Oct. 1998, and Ziermann et al., 1999, Recombinant  
polyketide synthesis in *Streptomyces*: engineering of improved host strain,  
20 *BioTechniques* 26: 106-110, both of which are incorporated herein by reference)  
35 transformants were prepared according to standard methods (see Hopwood et al.,  
1985, *supra*) using apramycin (100 µg/mL), thiostrepton (50 µg/mL), and  
hygromycin (225 µg/mL) in the R5 protoplast regeneration plates. In the three-  
40 plasmid system for which *eryAII*/pSAM2-hyg replaced *eryAII*/SCP2\*-hyg, *S.*  
25 *lividans* was transformed sequentially with pKOS010-153, pKOS038-67, and  
pKOS021-30.

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Example 4Diketide Feeding

The (2*S*, 3*R*)-2-methyl-3-hydroxyhexanoyl-*N*-acetylcysteamine (NAC) thioester (propyl diketide) was synthesized in accordance with the method described in U.S. patent application Serial Nos. 60/117,384, filed 27 Jan. 1999, and Serial No. 09/492,733 (attorney docket no. 30062-20032.00), filed 27 Jan. 00, by the same inventors and claiming priority to the foregoing application. The *Streptomyces lividans* triple transformants containing the KS1 null allele of *eryAI* (see Jacobsen *et al.*, 1997, *supra*, and PCT patent publication Nos. 99/03986 and 97/02358, each of which is incorporated herein by reference) were cultured in 5 mL of R5 medium at 30°C for 6 days under appropriate antibiotic selection. On day 4 of the incubation, 300 µL of diketide solution (4.7 mg/mL in 10% DMSO) and 50 µL of pentanoic acid (2.5 mg/mL) were added to the culture.

Example 5Production and Analysis of Polyketide Analogs

The *Streptomyces lividans* triple transformants were cultured in 5 mL of R5 medium (see Hopwood *et al.*, 1985, *supra*) at 30°C for 6 days under appropriate antibiotic selection. The cultured solution was extracted with 2 x 5 mL of ethyl acetate and the organic layers were combined and concentrated. A 50 µL aliquot of the concentrates was analyzed by HPLC on a reverse phase C18 column (4.6 mm x 15 cm, Beckman, Fullerton, CA) using a PE SCIEX API100 LC/MS based detector (Perkin-Elmer, Foster City, CA). Quantitative determination of polyketide yield was made with evaporative light scattering detection (Analtec model 500 ELSD, Deerfield, IL). The polyketides were identified by their mass spectrum and correspondence to the products expected or to known standards. Under the ionization conditions used, 6dEB and its analogs generate signature dehydration patterns. The yield of the 13-ethyl 6dEB analogs varied from 7 mg/L to less than 0.1 mg/L and the 13-propyl analogs were produced in a range of 0.2 to 20 mg/L. The yields of the 12-membered lactones were not determined.

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The invention having now been described by way of written description and examples, those of skill in the art will recognize that the invention can be practiced in a variety of embodiments and that the foregoing description and examples are for purposes of illustration and not limitation of the following

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5 claims.

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## Claims

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Claims

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1. A method for expressing a polyketide or non-ribosomal peptide in a host cell employing a multiplicity of recombinant vectors, which may be integrative or freely replicating, wherein each of the multiplicity of vectors encodes a portion of the polyketide synthase or non-ribosomal peptide synthase that produces the polyketide or non-ribosomal peptide, said method comprising steps of introducing said vectors into said cell, and culturing said cell into which said vectors have been introduced under conditions such that said polyketide synthase or non-ribosomal peptide synthase is produced.

10

20

2. The method of Claim 1, wherein said vectors encode a polyketide synthase.

25

3. The method of Claim 2, wherein said polyketide synthase is selected from the group consisting of DEBS, an oleandolide PKS, a picromycin PKS, an epothilone PKS, a megalomicin PKS.

30

4. The method of Claim 1, wherein said vectors encode a non-ribosomal peptide synthase.

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35

5. The method of Claim 1, wherein at least one of the multiplicity of vectors encodes a protein that further modifies the polyketide or non-ribosomal peptide produced by said polyketide synthase or non-ribosomal peptide synthase.

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6. The method of Claim 1, wherein at least one of said vectors replicates extrachromosomally in said host cell.

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7. The method of Claim 1, wherein at least one of said vectors integrates into the chromosome of said host cell.

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- 5                   8.     The method of Claim 1, wherein said host cell is a *Streptomyces* host cell.
- 10                   9.     The method of Claim 8, wherein said vector is a derivative of a  
5     vector selected from the group consisting of integrating vectors pSET152 and pSAM2 and extrachromosomally replicating vectors SCP2\* and pJV1.
- 15                   10.    A compound selected from the group consisting of compounds shown in Figure 3 as compound nos. 29-43 and 45-59.
- 10                   11.    A compound selected from the group consisting of compounds  
20     obtainable by hydroxylation and/or glycosylation of compounds 29-43 and 45-59 of Figure 3.
- 25                   12.    The compound of Claim 11 that is a 14-membered macrolactone  
15     with a C-6 and/or C-12 hydroxyl and/or a C-3 and/or C-5 glycosyl.
- 30                   13.    The compound of Claim 12, wherein said glycosyl is either a  
   desosaminyl residue at C-5 or a cladinosyl residue at C-3 or both.
- 20                   14.    The compound of Claim 10 that is compound number 29 or 30 of  
35     Figure 3.
- 40                   15.    The compound of Claim 10 that is compound number 31 or 32 of  
25     Figure 3.
- 45                   16.    The compound of Claim 10 that is compound number 33 or 34 of  
   Figure 3.
- 50                   17.    The compound of Claim 10 that is compound number 35 or 36 of  
   Figure 3.

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18, The compound of Claim 10 that is compound number 37 or 38 of  
Figure 3.

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5 19. The compound of Claim 10 that is compound number 39 or 40 of  
Figure 3.

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20. The compound of Claim 10 that is compound number 41, 42, or 43 of  
Figure 3.

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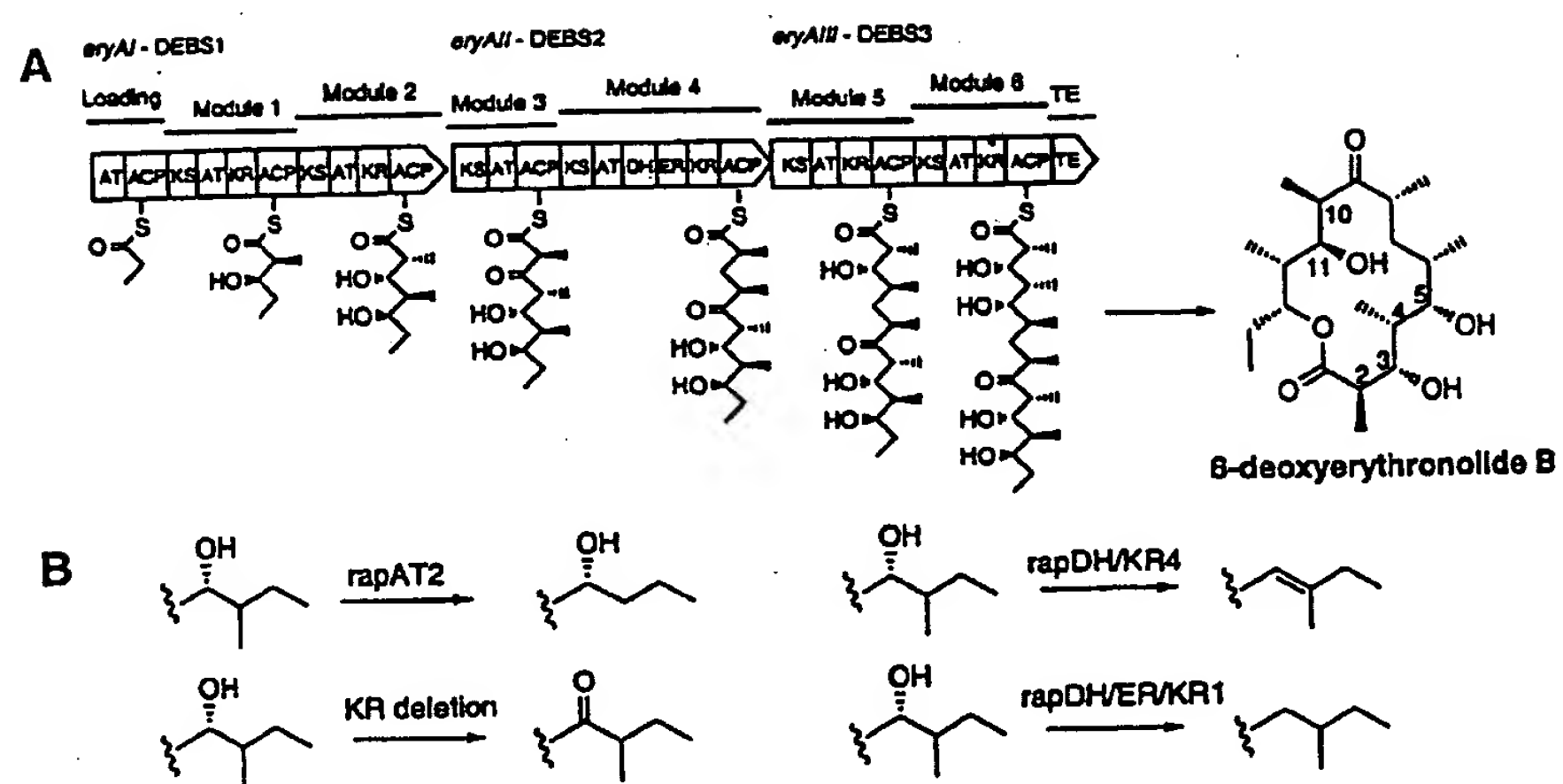
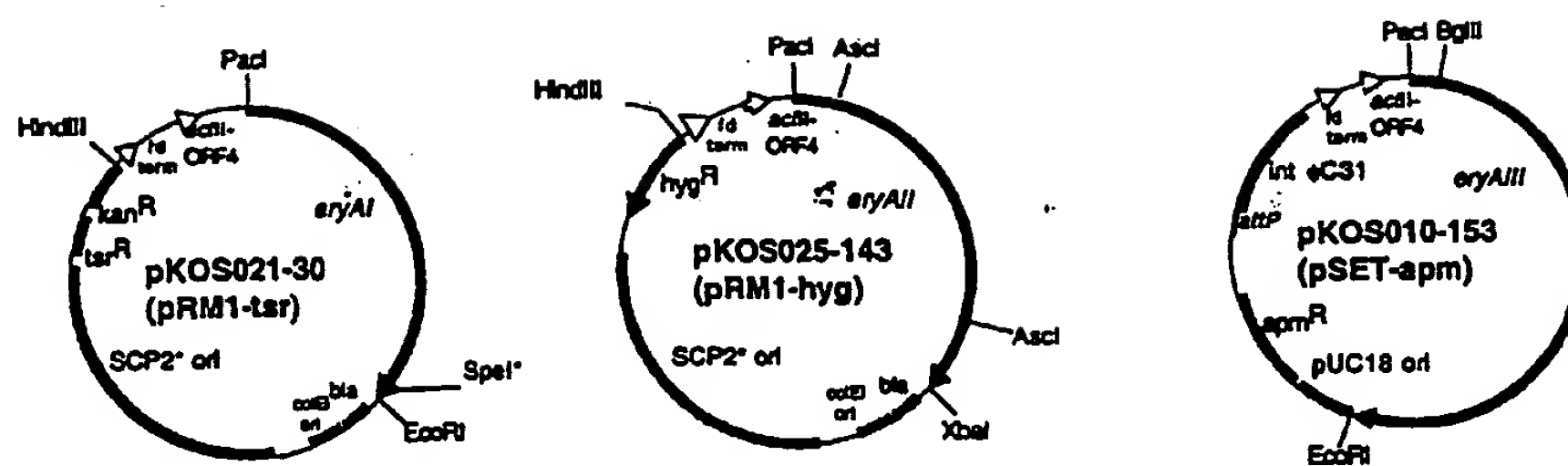


Figure 1



### Figure 2

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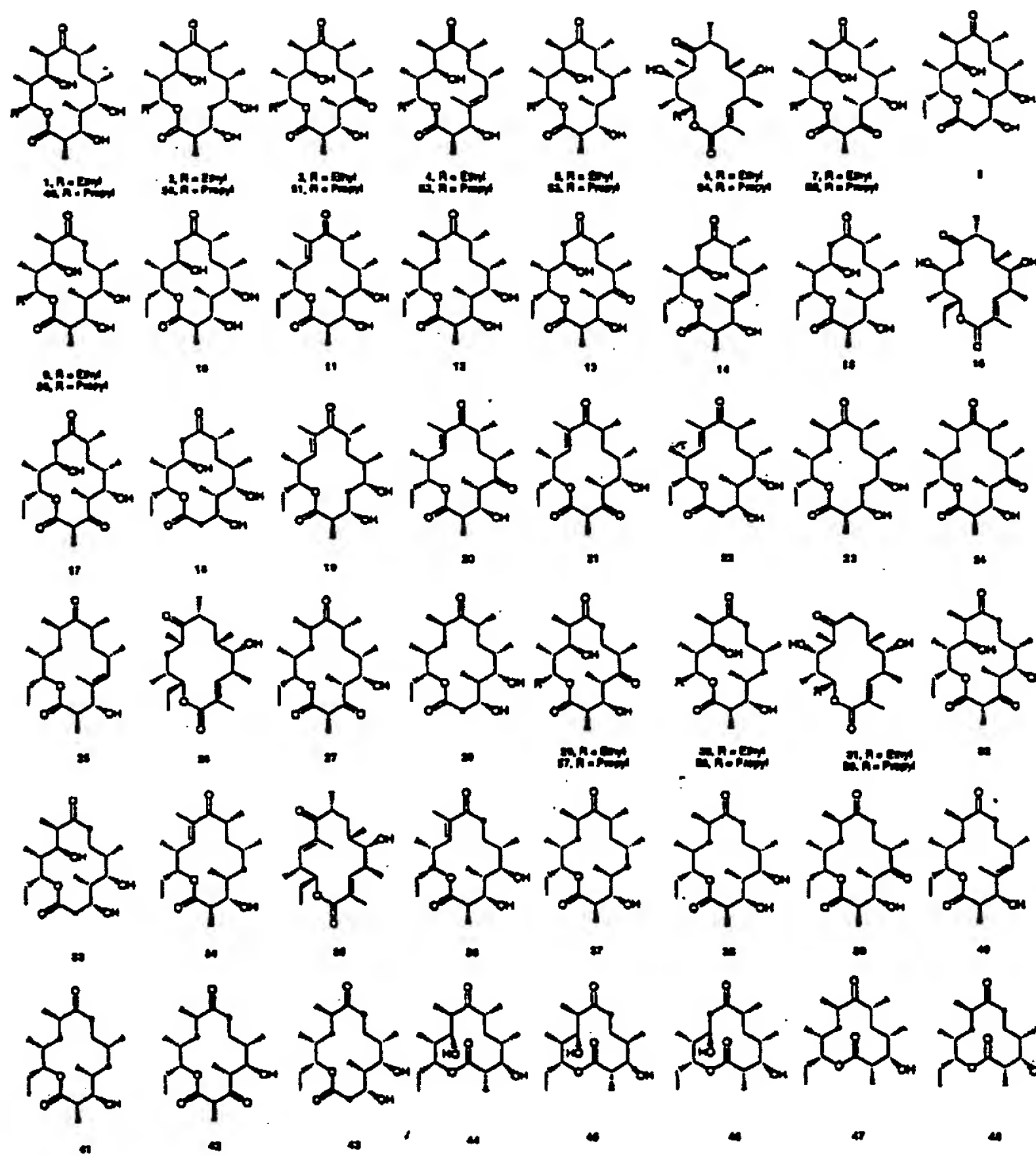


Figure 3